

EX-2

## Characterization of In Vitro Inhibition of Human Immunodeficiency Virus by Purified Recombinant CD4

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The first step in infection of human T cells with human immunodeficiency virus (HIV) is binding of viral envelope glycoprotein gp120 to its cellular receptor, CD4. The specificity of this interaction has led to the development of soluble recombinant CD4 (rCD4) as a potential antiviral and therapeutic agent. We have previously shown that crude preparations of rCD4 can indeed block infection of T cells by HIV type 1 (HIV-1). Here we present a more detailed analysis of this antiviral activity, using HIV-1 infection of the T lymphoblastoid cell line H9 as a model. Purified preparations of rCD4 blocked infection in this system at nanomolar concentrations; combined with the known affinity of the CD4-gp120 interaction, this finding suggests that the inhibition is simply due to competition for gp120 binding. As predicted, rCD4 had comparable activity against all strains of HIV-1 tested and significant activity against HIV-2. Higher concentrations of rCD4 blocked infection even after the virus had been adsorbed to the cells. These findings imply that the processes of viral adsorption and penetration require different numbers of gp120-CD4 interactions. Recombinant CD4 was able to prevent the spread of HIV infection in mixtures of uninfected and previously infected cells. Our studies support the notion that rCD4 is a potent antiviral agent, effective against a broad range of HIV-1 isolates, and demonstrate the value of purified rCD4 as an experimental tool for studying the mechanism of virus entry into cells.

Infection by human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (2, 11, 21), is largely defined by its cellular receptor, CD4. A role for membrane glycoprotein CD4 in the process of infection was first proposed on the basis of a correlation between expression of CD4 on certain lymphocytes and their susceptibility to infection (17, 26). Direct involvement of this surface protein in infection is now established by the finding that anti-CD4 antibodies block infection by HIV (1, 8, 24, 30) and transfer of CD4 to previously CD4-negative cells confers infectability (3, 23). Furthermore, HIV envelope glycoprotein gp120 binds to CD4 with high affinity and forms stable complexes (20, 22, 25, 32).

Although gp120 shows extensive genetic variation between HIV strains, potentially complicating the development of effective vaccines, all strains appear to bind to monomeric receptor CD4. This implies that at least one region of gp120 retains a conserved function and thus represents an attractive, stable target for an antiviral agent. While structural studies, such as those defining the subregions of gp120 and CD4 involved in their interaction (3, 4, 18-20, 27, 29), may eventually facilitate development of useful synthetic blocking agents (16), the most direct approach is to exploit the known gp120-binding properties of CD4. Therefore, we (32) and others (6, 9, 10, 15, 34) have expressed soluble forms of recombinant CD4 (rCD4), have shown that rCD4 retains gp120-binding activity, and have demonstrated that soluble rCD4 can block infection by HIV in vitro.

In this investigation, we examined the ability of purified

soluble rCD4 to block genetically diverse isolates of HIV and examined the effects of rCD4 on viral adsorption and penetration by using temperature to dissect these stages of viral infection. Our results show that rCD4 is effective against a diverse range of HIV isolates and reveal that different concentrations of rCD4 are required to inhibit the different stages of viral entry, providing insight into the mechanisms by which rCD4 acts to block each stage of infection.

### MATERIALS AND METHODS

**Cells and virus.** All experiments were performed with the H9 cell line originally obtained from Robert Gallo (National Cancer Institute, Bethesda, Md.). HIV type 1 (HIV-1) strain IIIB (11), also obtained from R. Gallo, was propagated by the method of Vujcic et al. (35). HIV-1 strain 906 was isolated in our laboratory from a male European patient with HIV-related dementia whose only known risk factor was sexual contact with a woman from Chad. HIV-1 strain AL, isolated from a Haitian patient in Boston, Mass., was provided by David Ho while at Massachusetts General Hospital, Boston (14). HIV-1 strain RJ4029 was isolated in our laboratory from a Haitian male with Kaposi's sarcoma. HIV-2 strain LAV-2<sub>ROD</sub> was provided by Jean-Claude Chermann, Institute Pasteur, Paris, France (7). Virus stocks of strains IIIB, AL, RJ4029, 906, and LAV-2<sub>ROD</sub> were all grown in H9 cells. Tissue culture supernatants were harvested at peak infectivity and stored in aliquots at -70°C in 60% fetal bovine serum.

**rCD4.** The CD4 gene was modified to omit the transmembrane and cytoplasmic regions (32). The resulting 368-amino-acid glycoprotein was expressed in CHO cells and secreted.

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The nondenatured, glycosylated protein was purified to greater than 99% purity.

**RT assay.** The assay for HIV reverse transcriptase (RT) was performed as previously described (12, 28). Virus was concentrated from 1 ml of tissue culture supernatants by incubation with 0.5 ml of polyethylene glycol-0.4 M NaCl overnight at 4°C. Control experiments indicated that rCD4 did not affect the RT assay.

**Infection conditions.** Infection of H9 cells was performed as described previously (12). Briefly, 60  $\mu$ l of inhibitor or control medium was incubated with 100 50% tissue culture infective doses (TCID<sub>50</sub>) of HIV in 60  $\mu$ l for 1 h at 4°C, and then an additional 60  $\mu$ l of  $5 \times 10^6$  H9 cells per ml in complete medium (20% fetal bovine serum in RPMI 1640, 2 mM L-glutamine, 2  $\mu$ g of Polybrene per ml) was added. After 1 h, 150  $\mu$ l of this mixture containing cells, virus, and inhibitor was transferred to 2 ml of complete medium and cultured in 24-well plates at 37°C in 5% CO<sub>2</sub> for 7 days. These cell densities, incubation volumes, and virus concentrations were maintained in all experiments. On day 4, the cultures were split 1:1 with fresh medium or inhibitor-containing medium as indicated. On day 7, the cell culture supernatant was harvested and assayed for RT activity. One TCID<sub>50</sub> was defined as the amount of virus that produced significantly greater than background RT activity (greater than 10,000 cpm/ml) in this 7-day culture system. All culture experiments were performed in duplicate, and the average of the RT results was reported. Percent inhibition was defined as  $1 - (\text{average of duplicate experimental culture RT values} / \text{average of duplicate medium control culture RT values}) \times 100$ . Virus-free controls (H9 cells in medium) were included in each experiment and were always RT negative.

**Continuous inhibition of infection.** For continuous inhibition experiments, rCD4 was included at the preincubation, adsorption, and 7-day culture steps. The stated concentration indicates the rCD4 concentration during both the cell-virus-rCD4 adsorption and 7-day culture steps.

**Pretreatment inhibition of infection.** For pretreatment inhibitor experiments, virus and rCD4 were incubated at 4°C for 1 h. Subsequently, cells were added (1 h, 4°C), and then the 300- $\mu$ l mixture was washed with 12 ml of complete medium. The resulting cell pellet (in 100  $\mu$ l of medium) was suspended in 4 ml of complete medium (no inhibitor) and cultured in two 2-ml wells for 7 days. The stated rCD4 concentration refers to the concentration present only during the virus-rCD4-cell step.

**Postadsorption inhibition of infection.** For postadsorption inhibitor experiments, H9 cells and virus were first incubated in bulk at 4°C at the same cell number-virus-volume ratios as in the previous experiments. This suspension was then washed to remove nonadsorbed virus. Aliquots of the virus-cell suspension were distributed to tubes containing dilutions of rCD4. The stated rCD4 concentration refers to the concentration present at this step. After incubation for 1 h at 4°C, these 300- $\mu$ l virus-cell-rCD4 suspensions were diluted with 12 ml of cold complete medium, centrifuged, aspirated to a 100- $\mu$ l volume, and then suspended in 4 ml of complete medium and cultured for 7 days in two 2-ml wells.

**Postpenetration inhibition.** For postpenetration inhibitor experiments, virus was adsorbed to H9 cells at 4°C as before and then allowed to penetrate at 37°C for 1 h before aliquots of infected cells were incubated with rCD4 in 2-ml cultures. In these experiments, a single concentration of rCD4, 2  $\mu$ g/ml, was used, but it was added to the 2-ml culture at 1 h or 1, 2, 3, 4, 5, or 6 days postinfection. The concentration of rCD4 was maintained at the day 4 split of the culture, when

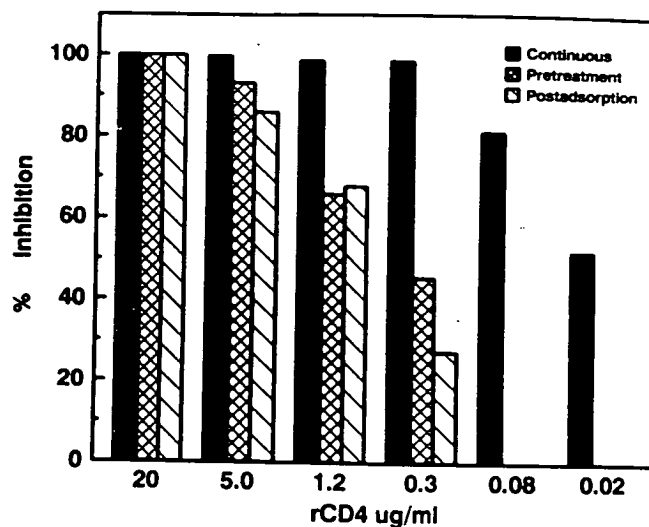


FIG. 1. Recombinant CD4 inhibition of infection by HIV-1 IIIB under continuous, pretreatment, and postadsorption conditions. For continuous-inhibitor conditions, the indicated concentrations of rCD4 were present throughout the 7-day culture period. The extent of infection was determined by measuring supernatant RT activity after 7 days in culture. See Materials and Methods for details of the infection procedure. The bars represent the mean results of three independent experiments. The mean positive control (no rCD4) RT value was  $3.8 \times 10^5$  cpm/ml. For pretreatment conditions, HIV-1 IIIB was preincubated with rCD4, target H9 cells were added, and after incubation, free rCD4 and virus were removed by washing. The cells were then cultured for 7 days. The bars represent the mean inhibition of RT activity in two separate experiments. The mean positive control was  $1.5 \times 10^5$  cpm/ml. For postadsorption conditions, HIV-1 IIIB was incubated with H9 cells at 4°C and free virus was removed by washing. rCD4 at the indicated concentrations was added to the HIV-cell complexes, incubated for 1 h at 4°C, and then removed by washing. The cells were then cultured for 7 days. The bars represent the mean inhibition of RT activity observed after 7 days in two separate experiments. The mean positive control RT value was  $1.5 \times 10^5$  cpm/ml.

appropriate, and left in culture until harvest on day 7. In another group of infected 2-ml cultures, rCD4 was added to a concentration of 2  $\mu$ g/ml at 1 h postinfection and then removed by washing of the culture and suspension of the cells in complete medium with no rCD4 at 1 h or 1, 2, 3, 4, 5, or 6 days postinfection.

**Cell-to-cell transfer of HIV.** H9 cells chronically infected with HIV-1 IIIB were mixed with uninfected H9 cells at a ratio of 1 infected cell to  $10^4$  uninfected cells. These mixtures were suspended in media containing 0, 0.01, 0.1, 1.0, or 10  $\mu$ g of rCD4 per ml. The mixtures were sampled every 2 to 3 days for supernatant RT activity and split to a density of  $2 \times 10^5$ /ml in 2 ml of the same CD4-containing medium (or control medium). On day 14, the cultures were washed free of rCD4 and suspended in medium containing no inhibitor. RT sampling continued until day 18.

## RESULTS

**Continuous inhibition.** Purified rCD4 blocked infection of H9 cells at concentrations as low as 20 ng/ml under continuous-inhibition conditions (Fig. 1). The lowest doses tested, 80 and 20 ng/ml, inhibited 82 and 52% of RT activity, respectively. These results were obtained in three separate experiments using two different virus stocks of HIV-1 IIIB

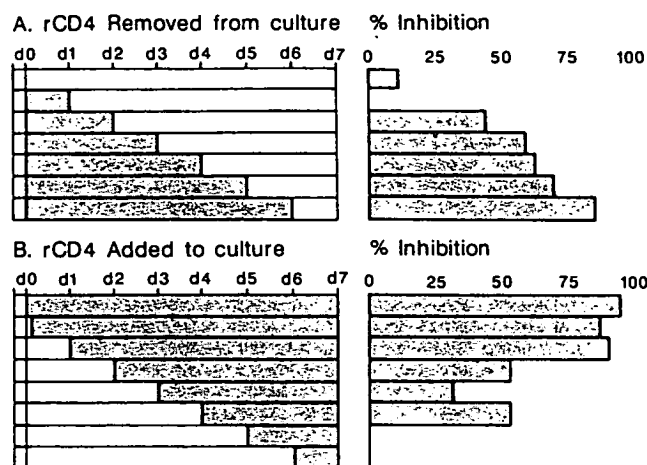


FIG. 2. Postinfection inhibition by rCD4. All H9 cultures were infected with 100 TCID<sub>50</sub> of HIV-1 IIIB. After the virus had been allowed to penetrate the cells for 1 h at 37°C, rCD4 was added. RT activity was assayed on day 7 in all cases. Shaded areas in the diagrams to the left indicate times during which 2  $\mu$ g of rCD4 per ml was maintained in the cultures. (A) rCD4 was added at day 0 and removed by washing and resuspending the cells in rCD4-free medium at the indicated times. (B) rCD4 was added to the cultures at the indicated times and maintained in the cultures until harvest on day 7. Percent inhibition under both conditions A and B was calculated by comparing test and control RT values. Controls ( $1.2 \times 10^5$  cpm/ml) were infected cultures to which no rCD4 was added. Each bar represents the mean of two cultures.

(each stock adjusted to 100 TCID<sub>50</sub> per culture). Soluble rCD4 had no effect on H9 cell viability, growth, or morphology in this culture system.

**Pretreatment with rCD4.** When higher concentrations of rCD4 were present during incubation of virus with cells at 4°C, infection was also blocked (Fig. 1). The concentration needed to inhibit infection by over 90% in these pretreatment experiments was approximately 20-fold higher than the inhibitory concentration in the continuous-inhibition system. The positive controls (100 TCID<sub>50</sub> of HIV-1 IIIB with no rCD4) resulted in slightly lower infection at the end of 7 days, indicating that washing the cells after virus binding slightly reduced infectivity. Preliminary experiments on virus adsorption at 4 and 37°C indicated that substantial binding occurred after 1 h at 4°C, but the level of saturation was lower than that observed at 37°C (data not shown). The results presented here were thus normalized to positive control cultures infected and washed under the same conditions as the experimental cultures. The conditions used to wash the cells after virus binding were designed to result in a 1/2,000 dilution of rCD4 in the final day 7 culture medium relative to its initial concentration. The results shown in Fig. 1 indicate that this level of carryover of rCD4 should not contribute significantly to the final result, even at the highest pretreatment rCD4 concentrations tested.

**Postadsorption inhibition.** When rCD4 was added to cultures after virus was adsorbed to H9 cells at 4°C, significant inhibition of infection was observed (Fig. 1), but at concentrations higher than before. A concentration of 5  $\mu$ g/ml was required for greater than 80% inhibition using HIV-1 IIIB stocks (100 TCID<sub>50</sub> per culture). rCD4 was thus able to inactivate or release HIV-1 from the cell surface, indicating that adsorbed virus was still accessible to added rCD4 and did not penetrate the cell membrane under these low-temperature conditions.

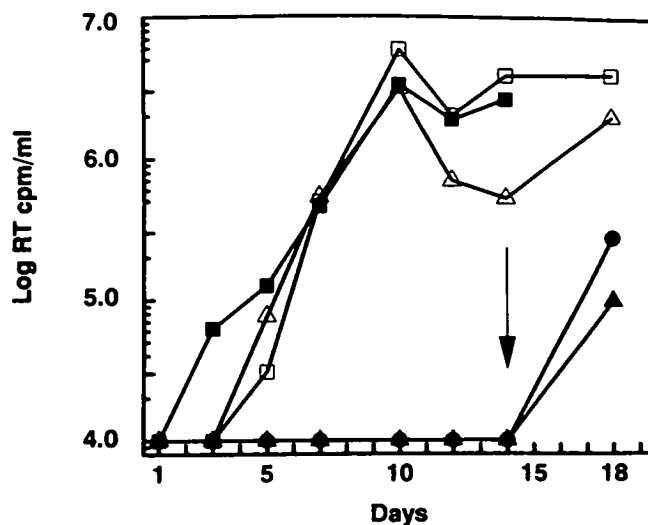


FIG. 3. Inhibition of cell-to-cell spread of HIV-1 by rCD4. Chronically infected H9 cells (HIV-1 strain IIIB) were mixed with uninfected cells at an infected-to-uninfected-cell ratio of 1:10<sup>4</sup> in the presence of control medium (■), 0.01  $\mu$ g of rCD4 per ml (△), 0.1  $\mu$ g of rCD4 per ml (□), 1.0  $\mu$ g of rCD4 per ml (▲), or 10.0  $\mu$ g of rCD4 per ml (●). On day 14 (arrow), all cultures were washed to remove rCD4 and suspended in control medium.

**Postpenetration inhibition.** When rCD4 was added to previously infected H9 cell cultures (100 TCID<sub>50</sub> of HIV-1), significant inhibition was observed, as measured by reduction in RT activity at the end of the 7-day culture period (Fig. 2). The greatest inhibition (>80%) was observed when rCD4 was added within the first 2 days of infection and maintained throughout the culture period. Conversely, when rCD4 was present only during the first or last 2 days of the 7-day culture period, little inhibition (<50%) was observed.

**Inhibition of cell-to-cell transfer of HIV.** HIV spread rapidly from chronically infected cells to uninfected cells in cultures containing control medium or low concentrations, 0.01 or 0.1  $\mu$ g/ml, of CD4 (Fig. 3). Cultures containing 1.0 or 10  $\mu$ g of rCD4 per ml showed no evidence of spread of infection during the initial 14-day period. At this time, however, infected cells were still present in the 1.0- and 10- $\mu$ g/ml rCD4 cultures, because removal of rCD4 allowed rapid spread of infection with kinetics similar to the that of the initial control medium culture.

**Inhibition of HIV-1 and HIV-2 strains.** Earlier reports (9, 10, 15, 32, 34) of HIV-1 inhibition by rCD4 focused on a single HIV-1 strain, IIIB. To confirm the inhibitory effect of rCD4 against multiple isolates of HIV (6), we performed the following experiment. The titers of three additional HIV-1 strains, all confirmed to be significantly different by restriction endonuclease mapping and partial nucleic acid sequence determination (data not shown), were determined in our 7-day H9 cell system, and 100-TCID<sub>50</sub> challenge inocula were prepared. With the continuous-inhibition format, rCD4 inhibition curves were obtained for the different strains (Fig. 4). A similar experiment was performed with HIV-2 strain LAV-2<sub>ROD</sub> (7). Each curve presented in Fig. 4 is the mean of at least two experiments. While some HIV-1 strains showed reproducible differences in sensitivity to inhibition, the shapes of the titration curves were similar for all four HIV-1 isolates tested. In contrast, HIV-2 showed both an inhibition curve shape and a sensitivity to low rCD4 concentrations that appeared to be different from those of HIV-1.

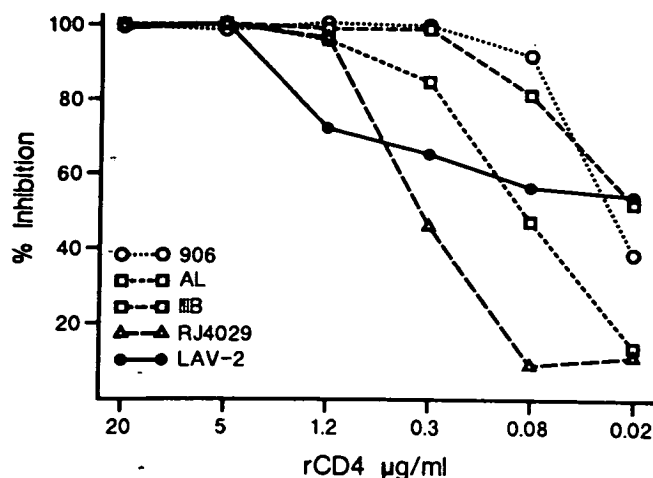


FIG. 4. rCD4 inhibition of infection by HIV-1 strains and HIV-2. H9 cells were infected with 100 TCID<sub>50</sub> of each viral strain in the presence of different concentrations of rCD4 (continuous-inhibition conditions; see Materials and Methods). Infection was monitored by measuring supernatant RT activity after 7 days in culture. The results for HIV-1 IIIB are those presented in Fig. 1. HIV-1 isolates are 906, AL, IIIB, and RJ4029. LAV-2 is an HIV-2 isolate. RT activities in the positive controls were as follows: 906,  $1.0 \times 10^6$  cpm/ml; AL,  $1.4 \times 10^6$  cpm/ml;  $3.8 \times 10^5$  cpm/ml; RJ4029,  $6.8 \times 10^5$  cpm/ml; LAV-2,  $2.6 \times 10^5$  cpm/ml.

## DISCUSSION

We have shown that rCD4 inhibited infection by free HIV-1 in vitro at a concentration of 0.3 µg/ml (7 nM) when inhibitor and virus were added together (Fig. 1). Indeed, when the virus was preincubated with rCD4 and rCD4 was present throughout the culture period, concentrations as low as 20 ng/ml (0.5 nM) provided significant protection from infection. Since the interaction between gp120 and either cell surface or recombinant CD4 has a dissociation constant of 1 nM, this indicates that the mechanism involved in virus inhibition is probably direct competition for gp120 binding, as previously proposed (32). Since we measured only infection of cells, not virion binding, we cannot exclude the possibility that virions are directly inactivated by rCD4; however, we consider it more likely that inhibition of viral adsorption is the major, and possibly the only, mechanism by which rCD4 inhibits infection by free virus. rCD4 has previously been shown to block adsorption of virus to cells (9). In addition, the finding that HIV-1 virions shed their gp120 readily (31) suggests that CD4 inactivates HIV simply by sequestering virion-bound gp120 until it is lost from the virus, rendering the virion uninfected. If this process does occur, the results of our pretreatment experiments (Fig. 1) could be interpreted to indicate that dissociation of rCD4 from virus is slow relative to the time for which the virus remains infectious.

Once the virus has adsorbed to a cell, the picture is more complicated. We took the approach of attempting to dissect the process of infection by using temperature: H9 cells were productively infected with HIV-1 at 37°C but not at 4°C, although cells given virus at 4°C became productively infected when warmed, suggesting that adsorption, but not infection, can occur efficiently at 4°C. Indeed, rCD4 at concentrations of 5 µg/ml can block infection even when the virus has previously been allowed to adsorb to the cells at 4°C (Fig. 1). While previous results have suggested that penetration by HIV is independent of temperature, based on

electron microscopy (33), our results suggest that the reaction is likely to be slowed, if not entirely stopped. Recently, Clapham et al. (6) have shown postadsorption inhibition of infection by HIV-1 on monolayers of C8166 cells. Their reduction in HIV-1 titer, approximately 30-fold, measured at a single rCD4 concentration of 40 µg/ml, is slightly lower than that observed in our system, but the difference could be due to a number of experimental variables.

The observed postadsorption inhibition of infection could be due to (i) release of virions from the cell surface or (ii) inhibition of penetration and uncoating. We suggest that the finding can be explained if a small number of gp120-CD4 interactions is sufficient to attach a virion to the surface of the cell but many such interactions are required to initiate membrane fusion. The process of virus attachment and infection would then be analogous to a two-dimensional zipper or a piece of Velcro. rCD4 could inhibit membrane fusion by covering the teeth of the zipper, lowering the probability that the high density of interactions required for virus penetration can occur. If gp120-CD4 interactions are also necessary to the progression of fusion, rCD4 could block this progression even after fusion has been initiated.

Even after the initial infection by virions is completed, rCD4 can inhibit the spread of infection (Fig. 2). Infection in such cultures can spread either by cell-to-cell contact or by release of new virions, which in our system occurs within 24 to 36 h after inoculation (16a). Since both of these mechanisms should be blocked by rCD4, our present results do not distinguish between the two mechanisms.

The primary difference between cell-to-cell spread of infection and free-virion infection may be that infected cells produce virus near the target with respect to both space and time, so the inoculum is essentially fresh and concentrated. In our cell-mixing system (Fig. 3) 1 µg of rCD4 per ml blocked the spread of infection. The similarity of this effective dose level to that observed for free-virion infection (Fig. 1) suggests that infection of H9 cell cultures does not involve cell fusion pathways insensitive to rCD4 inhibition. Analysis of other, more fusogenic cell types may yield different results.

Preliminary experiments by several groups, including ours, have shown inhibition of HIV-1 strain IIIB by soluble rCD4 (9, 10, 15, 32, 34). The results presented here confirm that rCD4, as recently reported (6), is not a type-specific inhibitor, since it can block infection by HIV-1 strains quite different from IIIB (for example, HIV-1 AL has 83.5% amino acid identity to HIV-1 IIIB; D. Dowbenko, personal communication) and by HIV-2 (39.4% amino acid identity to HIV-1 IIIB) (13). The inhibition curves for the various HIV-1 strains had similar slopes, suggesting that the affinities of the various gp120 molecules for rCD4 are comparable. Because the sizes of the viral inocula used in these experiments were determined by using the infectious titer of the virus rather than the total amount of gp120 or the total number of virions, the amount of gp120 in the inocula may vary significantly. Analysis of our viral inocula by direct RT activity measurement, p24 antigen content measurement (Abbott Laboratories), and Western blotting (immunoblotting) using high-titered anti-HIV-1 sera from patients indicated that the ratio of infectious units to total viral material was highest for HIV-1 IIIB, 906, and LAV-2 and significantly lower for AL and RJ4029 (data not shown). Exact determination of the gp120 content of each inoculum, perhaps the most critical parameter, was not performed because all available methods of gp120 detection rely on immunologic reagents that, in turn, can be expected to vary in sensitivity

with the antigenic character of the strain being tested. These differences in infectivity-to-viral protein ratios, rather than affinity differences, are the most likely explanation for the observed differences in sensitivity to rCD4 inhibition among the HIV-1 strains tested.

The rCD4 inhibition curve for HIV-2 strain LAV-2 is clearly different from those of the HIV-1 strains, requiring higher rCD4 concentrations for full inhibition and displaying a shallower slope. The reason for this difference is not clear, but a difference would be expected if HIV-2 gp120 had a lower affinity for CD4 (whether recombinant or cell surface) or if HIV-2 virions had a higher density of gp120 on their surface.

Our results may have relevance to the therapeutic application of soluble rCD4, since the concentrations of H9 cells in these *in vitro* experiments are comparable to those of CD4<sup>+</sup> cells in the bloodstream. The levels of rCD4 required in our assays to block HIV-1 infection completely are probably achievable in the blood of infected patients, as suggested by half-life studies with animal models (5). Furthermore, our results strongly support the idea that rCD4 will be effective in blocking infection by most or all strains of HIV and in blocking the spread of infection both by free virions and by cell-to-cell contact.

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